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(54) Title: NOVEL SYNTHETIC GRF ANALOGS

(57) Abstract

The present invention relates to novel peptides having therapeutic utility. More specifically, the invention relates to peptide analogs of the naturally occuring peptide, growth hormone-releasing factor (GRF). The invention also relates to therapeutic compositions containing these peptides, and therapeutic methods using these peptides to stimulate the productioin of growth hormone *in vivo*.

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NOVEL SYNTHETIC GRF ANALOGS

1. FIELD OF THE INVENTION

The present invention relates to novel peptides having therapeutic utility. More specifically, the invention relates to peptide analogs of the naturally occurring peptide, growth hormone-releasing factor (GRF). The invention also relates to therapeutic compositions containing these peptides, and therapeutic methods using these peptides to stimulate the production of growth hormone *in vivo*.

2. BACKGROUND OF THE INVENTION

Growth hormone (GH or somatotropin) is a 191-amino acid peptide which is secreted by the anterior pituitary. Growth hormone itself does not actually promote growth directly but acts by simulating the production of one of the many true growth factors such as the somatomedins produced by the liver. The ultimate effects of growth hormone are widespread however. On a gross level, this hormone affects the skeleton, connective tissue, muscles and viscera. On a molecular level, the metabolic effects of growth hormone and somatomedins include stimulation of nucleic acid and protein synthesis, induction of positive nitrogen balance, stimulation of lipolysis, and a decrease in urea excretion.

Inadequate levels of growth hormone in children causes retardation of growth, epiphyseal development and bone age. It also causes retarded development of secondary sexual characteristics. Additional effects of growth hormone deficiency include impaired larynx development, delayed gonadal maturation, and hypoglycemia. These effects can all be reversed with normal levels of growth hormone.

The production of growth hormone is under the control of both releasing and inhibitory influences located in the hypothalamus. The primary releasing influence is growth hormone releasing factor (GRF or GHRH), which is produced primarily in the arcuate nucleus of the hypothalamus, and is transported to the pituitary by portal circulation. However, other cells of the body such as pancreatic tumor cells, may also produce this hormone. Growth hormone releasing factor in humans is a peptide 44 amino acids in length of which the first 29 contain the full biological activity. Similar peptides have also been isolated from cow, rat, sheep and pig, and their sequences identified (Esch et al., Biochem. Biophys. Res. Commun. 117:772, 1983).

The discovery of the sequence of GRF has provided a physiologically natural means for treating individuals with growth hormone deficiencies. Substantial efforts have been devoted to development of synthetic analogs of GRF in the hope that such analogs will be more efficient in causing release of growth hormone.

Efforts at creating new substitutions have focused on the N-terminal portion of the molecule, particularly residues 1-29, in which the biological activity lies. Various substitutions in one or more of the amino acid residues have been shown to be effective in increasing the potency of the synthetic peptides relative to that of the natural peptide. One current approach in peptide design generally has been to explore modifications which will enhance the amphiphilic secondary structure of the peptide (De Grado et al., J. Am. Chem. Soc. 103:679-686, 1981); Mue et al., J. Am. Chem. Soc. 105:4100-4102, 1983). Certain peptide hormones, i.e., those in which one face of the molecule has preferentially hydrophobic residues, while the other face contains a primarily hydrophilic domain, may have their activity enhanced by optimization of this amphiphilic

character. GRF has been noted as having substantial amphiphilic potential, based on observations of its binding to single bilayer phospholipid vesicles, and formation of a monolayer at the air-water interface (Kaiser et al., Science 223:249-255, 1984).

A number of attempts to alter GRF secondary structure have been reported in the literature. For example, Lance et al. (Biochem. Biophys. Res. Comm. 119:265, 1984) attempted to build conformational restriction into the N-terminus of the molecule by replacement of L-amino acids in positions 1, 2 and 3 (Tyr^1, Ala^2, Asp^3) with their D-isomers. It was found that each of the D-analogs was more active than the native peptide. Subsequent studies extended this approach to positions 4, 5, 6, 7 and 8. Substitutions at positions 5, 6, and 7 resulted in substantial loss of activity, whereas D-substitution at position 4 retained similar activity and at position 8 increased activity (Coy et al., J. Med. Chem 28:181-185, 1985).

Further attempts to modify the secondary structure of the basic GRF molecule have focused on attempting to enhance the proposed amphiphilicity at certain other points on the molecule. For example, Tou et al., (Biochem. Biophys. Res. Comm. 139:763-770, 1986) noted the predicted positions of α-helicity on the GRF (1-29) molecule, and modified certain regions of the molecule by substituting certain amino acids in the 13-29 region as well as in positions 6-10 in which a β-turn is expected to exist. Replacement of Ser at position 9 with an Ala residue (a "good α-helix former") was said to favor an extended helical conformation. All peptides created with this pattern exhibited enhanced activity relative to the native GRF.

It has now been surprisingly discovered that the substitution of L-Ala, or equivalent amino acid residues, at position 8 of the native molecule brings about an enormous increase in the growth hormone releasing activity of

peptides so modified. Although other substitutions at the number 8 position have previously been suggested (see e.g. U.S. Patent No. 4,518,586, in which D-Arg, Ser, and D-Ser are substituted; U.S. Patent No. 4,689,318, in which Ser, Asn, Thr or Gln are substituted); U.S. Patent No. 4,626,523, in which D-Arg or D-Lys are substituted) no one has proposed the use of L-Ala at this position. Indeed, Saito et al. (Biochem. Biophys. Res. Comm. 149:531-537, 1987) substituted a D-Ala at this position, and found a substantial decrease in activity. Thus, the enhancement observed with the present specific substitution is particularly surprising.

3. SUMMARY OF THE INVENTION

The present invention relates to a group of novel peptides which are analogues of the native hormone GRF. The novel hormones are characterized by substitution of the Asn normally at the number 8 position in the native molecule with amino acids which are conducive to α-helix formation. Specifically, the invention relates to peptides comprising the formula: R_1 - R_2 - R_3 - Ala - Ile - Phe - R_7 - R_8 - R_9 - R_{10} - Arg - R_{12} - R_{13} - R_{14} - R_{15} - Gln - R_{17} - R_{18} - Ala - Arg - R_{21} - Leu - R_{23} - R_{24} - R_{25} - R_{26} - R_{27} - R_{28} - R_{29}

wherein R_1 is des-amino-Tyr, or A-R_1 , in which A is lower alkyl, lower cycloalkyl, benzyl or lower acyl and R_1 is Tyr, D-Tyr, Met, Phe, D-Phe, pCl-Phe, Leu, His, or D-His with or without a $C^{\alpha}Me$ or $N^{\alpha}Me$ substituent

R_2 is Ala, D-Ala, D-NMA, or D-Arg;

R_3 is Asp or D-Asp;

R_7 is Thr, Aib, NO_2, Leu, Trp, β-Nal, or p-X-Phe, in which X = H, F, Cl, Br, NO_2, or Me;

R_8 is Ala, Aib, Leu, Trp, β-Nal, or p-X-Phe, in which X is H, F, Cl, Br, NO_2, or Me;

R_9 is Ser, Ala, Aib, Leu, Trp, β-Nal or p-X-Phe, in which X is H, F, Cl, Br, NO_2, or Me
R_{10} is Tyr or D-Tyr
R_{12} and R_{21} are Lys, Arg, or N^{ξ}-B-Lys, in which B is lower alkyl or cycloalkyl, and may be the same or different
R_{13} is Ile or Val
R_{14} is Leu or D-Leu
R_{15} Gly, Ala, Leu, Asn, Gln or Aib
R_{17} is Leu or D-Leu
R_{18} is Tyr or Ser
R_{23} is Leu or d-Leu
R_{24} His or Gln
R_{25} is Glu, Asp, D-Glu or D-Asp
R_{26} is Ile or Leu
R_{27} is Met, D-Met, Ala, Nle, Ile, Leu, Nva, or Val
R_{28} is Asn, Ser or des R_{28}
R_{29} is Arg, D-Arg or des R_{29};
and pharmaceutically acceptable salts thereof.

In a preferred embodiment, R_8 is Ala; in a particularly preferred embodiment, R_8 and R_{15} are Ala, and most preferably, R_8, R_9, and R_{15} are Ala. A particularly effective peptide is formed when R_8, R_9, and R_{15} are Ala, and R_2 is D-Ala.

Although the formula stated above contains 29 amino acid residues, it will be understood by those skilled in the art that this represents the active portion of the molecule, and that addition of further amino acid residues to the C-terminus of the sequence will not affect the efficacy. Additions bringing the total up to 44 residues, as are present in the native GRF molecule, may be made without effect. Since these amino acids are not essential to activity, their identity is not critical; they may be the

same as in the natural GRF sequence, or reasonable equivalents. Such alternate substitutions are found in, for example U.S. Patent Nos. 4,626,523 and 4,728,726.

The invention also provides compositions comprising a growth hormone-releasing effective amount of the peptides/salts described above in combination with a pharmaceutically acceptable carrier. Also provided is a method of stimulating release of growth hormone by administration of a composition of the invention to an individual in need of such treatment. Such a method is useful in the treatment of physiological conditions in which release of a growth hormone would be expected to be of benefit. A method of stimulating growth in healthy animals is also provided which comprises administering the compositions of the present invention to such animals.

In an alternate embodiment, a particularly effective GRF receptor antagonist is produced by combining the specified substitutions at the 8, 9, or 15 positions with a substitution of D-Arg in the R_2 position. Such peptides inhibit the activity of endogenous GRF, and therefore in turn prevent the release of growth hormone. Thus, also provided are therapeutic compositions comprising a GRF-antagonistic effective amount of such a peptide, in combination with a pharmaceutically acceptable carrier as well as methods for inhibiting release of growth hormone by administering these compositions to an individual in need of such treatment.

4. ABBREVIATIONS

The following abbreviations are used throughout the specification and claims.

Ala	Alanine
Arg	Arginine
Asn	Asparagine
Cys	Cysteine
Gln	Glutamine
Gly	Glycine
His	Histidine
Ile	Isoleucine
Leu	Leucine
Lys	Lysine
Met	Methionine
Phe	Phenylalanine
Pro	Proline
Ser	Serine
Thr	Threonine
Trp	Tryptophan
Tyr	Tyrosine
Val	Valine
Aib	α-amino butyric acid
des-amino-Tyr	[beta(para-hydroxyphenyl)propionyl]
Nle	norleucine
Nva	norvaline
β-Nal	β-naphthylalanine
D-NMA	D-alanine substituted with methyl at the alpha-amino group
BOC	t-butyloxycarbonyl
Bzl	benzyl
Tos	P-toluenesulfonyl
2-Cl-Z	2-chlorobenzyloxycarbonyl
2-Br--Z	2-bromobenzyloxycarbonyl
chx	cyclohexyl

In all cases in which isomeric forms of an amino acid exist, where no letter precedes a named residue, the naturally occurring L-isomer is intended. Also, unless a specific C-terminus substituent is noted, both the -OH (free acid) and $-NH_2$ (amide) forms of the peptide are contemplated.

Also, as used herein, the terms "lower" in "lower alkyl" "lower acyl" and "lower cycloalkyl" refers to C 3-4, and "cycloalkyl" is C 3-6.

5. DETAILED DESCRIPTION OF THE INVENTION

The peptides of the present invention are analogues of native GRF which exhibit greater potency than the natural hormone in releasing growth hormone. The increase in potency of these analogues is believed to be due to an enhancement in the α-helical properties of the N-terminal portion of the GRF molecule. Although it has previously been shown that enhancement of the amphiphilic α-helical properties of the C-terminal region results in increased potency of the peptides, it was not known how such modification on the N-terminus would affect the activity of the molecule. The present results show that an unexpected increase in potency is obtained by substitution at position 8 in GRF [1-29] with amino acid residues having properties which tend to increase α-helicity. Among the amino acids in this category are Ala, Aib, Leu, Trp, β-Nal, or p-X-Phe, in which X is H, F, Cl, Br, NO_2, or Me. Most preferably, R_8 is Ala. Analogues in which the only modification of the GRF [1-29] sequence is substitution of Ala^8 for Asn^8 have a potency of at least 4 times that of the native molecule in vitro (Table 1). Substitution at position 15 of Ala, Leu, Asn, Gln, or Aib, preferably Ala, results in even greater increase in potency, up to about 5 times that of native GRF in vitro. A further favorable modification is the substitution at position 9 of one of the same preferred

residues used at position 8. The combination of these substitutions at the 8, 9, and 15 position, particularly when they are all Ala substituents, enhances the potency, and when combined with Glu^{25} and $Leu^{26,27}$, activity is increased to 33 times that of the native GRF molecule.

It has previously been disclosed that the substitution of D-Ala at the 2 position results in increased activity of GRF [1-29]. In a particularly preferred embodiment of this invention, this substitution is combined with one or more of the 8, 9, or 15 substitutions to produce a highly potent GRF analogue having up to 49 times the activity of native GRF in vitro.

For enhancement of GRF antagonist activity, the proposed substitutions at the 8 and/or 9 and/or 15 positions, with peptides having Arg at the 2 position are particularly effective. Although the use of the Arg^2 substitution has previously been reported to result in GRF antagonist activity, the proposed combinations result in even greater antagonistic activity, up to 10 times that of the D-Arg^2 substitution alone.

5.1. METHOD OF PREPARATION

The preparation of the peptides of the present invention can be accomplished by any of the known methods for peptide synthesis. A preferred method is solid phase synthesis such as described by Merrifield (J. Am. Chem. Soc. 85:2149, 1963). In this method, construction of the peptide begins at the C-terminus; an amino-protected amino acid is coupled, by known methods, to an appropriate resin, such as a chloromethylated resin, a benzhydryl-amine resin or a methylbenzhydrylamine resin. Once coupling has occurred, deprotection of the terminal amino acid is performed in accordance with techniques well known to those skilled in the art. A typical deprotection medium comprises trichloroacetic acid, either alone or in combination with

methylene chloride. The remaining protected amino acids of the desired sequence are added successively in the appropriate order to the solid phase, and coupled to the preceding amino acid. Common coupling agents are carbodrimide derivatives, such as dicyclohexylcarbodiimide and diisopropylcarbodiimide. Alternately, several amino acids may be coupled to each other before being added to the solid phase synthesizer. When the sequence has been completed, the entire peptide is cleaved from the resin, and may be purified by routine procedures in the art, such as HPLC, or other chromatographic techniques.

5.2. PHARMACEUTICAL COMPOSITIONS

The synthetic peptides will often be used therapeutically in the form of their pharmaceutically acceptable salts, such as acid addition salts and metal complexes; such salts include hydrochloride, hydrobromide, sulfate, acetate, maleate, benzoate, citrate, tartrate, ascorbate, or phosphate, and metal complexes of iron or zinc.

The peptides are preferably administered parenterally, i.e., subcutaneously, intravenously, intramuscularly, intraperitoneally or percutaneously. For these modes of administration, the peptides will normally be combined with a pharmaceutically acceptable carrier, such as water, or isotonic saline. Alternatively, the peptides may be administered orally in the form of tablets or capsules, which contain the appropriate binders, lubricants and the like.

The amount of peptide needed per composition, and/or unit dosage can readily be determined by reference and comparison to known compositions and dosage forms currently in use for GRF peptides. In general terms, however, the dosage range will be between 50 ng - 5 μg/kg of body weight of the host. The dose will depend upon the mode

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of administration and the intended result. However, such manipulations are well within the ability of one skilled in the art.

5.3. THERAPEUTIC USES

The synthetic peptides are useful in any situation in which direct administration of growth hormone would be desired. For veterinary use, this includes for example administration to livestock, such as cattle, chickens, turkeys, pigs, goats, fish, and the like, both to promote growth and also to alter or improve the ratio of protein (muscle) to fat in such animals.

The peptides are also useful for the treatment of growth hormone deficiency-related disorders, such as pituitary dwarfism. Various other metabolic or developmental processes such as wound healing are also affected by growth hormone, and may thus benefit by administration of the present GRF analogues. Alternate uses of these peptides will be readily recognized by the skilled artisan.

Those peptides of the invention in which one or more of the proposed Ala substitutions are combined with Arg^2 are GRF antagonists and may therefore be used in treatment of conditions caused by excess growth hormone. An example of such a condition is acromegaly, which results in abnormal enlargement of the bones of the face and extremities, among other symptoms.

6. EXAMPLES

6.1. SYNTHESIS OF ANALOGUES

6.1.1. [Ala^8]-GRF(1-29)-methylbenzhydrylamine Resin

Benzhydrylamine-polystyrene resin (Advanced ChemTech, Inc.) (1.25 g, 0.5 mmole) in the chloride ion form is placed in the reaction vessel of an Advanced ChemTech peptide synthesizer programmed to perform the following reaction

cycle: (a) methylene chloride; (b) 33% trifluoroacetic acid in methylene chloride (2 times for 1 and 15 min each); (c) methylene chloride; (d) ethanol; (e) methylene chloride; (f) 10% disopropylethylamine in methylene chloride.

The neutralized resin is stirred with Boc-N^G- tosyl-Arg and diisopropylcarbodiimide (1.5 mmole each) in methylene chloride for 1 h and the resulting amino acid resin is then cycled through steps (a) to (f) in the above wash program. The following amino acids (1.5 mmole) are then coupled successively by the same procedure, except that Gln and Asn were coupled in the presence of 1.5 mmole of 1-hydroxybenzotriazole:

Boc-Ser(Bzl), Boc-Met, Boc-Asp(chx), Boc-Gln, Boc-Leu, Boc-Lys(2-Cl-Z), Boc-Arg(Tos), Boc-Ala, Boc-Ser(Bzl), Boc-Leu, Boc-Gln, Boc-Gly, Boc-Leu, Boc-Val, Boc-Lys(2-Cl-Z), Boc-Arg(Tos), Boc-Tyr(2-Br-Z), Boc-Ser(Bzl), Boc-Ala, Boc-Thr(Bzl), Boc-Phe, Boc-Ile, Boc-Ala, Boc-Asp, Boc-Ala, Boc-Tyr(2-Br-Z). After removal of the last Boc group and washing and drying, the completed resin weighed 2.5 g.

6.1.2. $\underline{Ala^8GRF(1-29)NH_2}$

The resin described in Section 9.1.1. (2.5 g, 0.5 mmole) is mixed with p-cresol (5 ml), dithiothreitol (100 mg) and anhydrous hydrogen fluoride (35 ml) at)°C and stirred for 45 min. Excess hydrogen fluoride is evaporated rapidly under a stream of dry nitrogen and free peptide precipitated and washed with ether. The crude peptide is then dissolved in a minimum volume of 2 M acetic acid and eluted on a column (2.5 x 100 cm) of Sephadex G-50 using the same solvent. Fractions containing a major component by uv absorption and thin layer chromatography are then pooled, evaporated to a small volume and applied to a column (2.5 x 50 cm) of Vydac octadecylsilane silica (10-15 μM).

This is eluted with a linear gradient of 10-45% acetonitrile in 0.1% trifluoroacetic acid in water. Fractions are examined by thin layer chromatography and analytical high performance liquid chromatography and pooled to give maximum purity. Repeated lyophilization of the solution from water gives the product as a white, fluffy powder.

The product is found to be homogeneous by HPLC and TLC. Amino acid analysis of an acid hydrolysate confirms the composition of the octapeptide.

6.1.3. [$Ala^{8,15}$]-GRF(1-29)-methylbenzhydrylamine Resin

Benzhydrylamine-polystyrene resin (Advanced ChemTech, Inc.) (1.25 g, 0.5 mmole) in the chloride ion form is placed in the reaction vessel of an Advanced ChemTech peptide synthesizer programmed to perform the following reaction cycle: (a) methylene chloride; (b) 33% trufluoroacetic acid in methylene chloride (2 times for 1 and 15 min each); (c) methylene chloride; (d) ethanol; (e) methylene chloride; (f) 10% diisopropylethylamine in methylene chloride.

The neutralized resin is stirred with Boc-N^G- tosyl-Arg and diisopropylcarbodiimide (1.5 mmole each) in methylene chloride for 1 h and the resulting amino acid resin is then cycled through steps (a) to (g) in the above wash program. The following amino acids (1.5 mmole) are then coupled successively by the same procedure, except that Gln and Asn were coupled in the presence of 1.5 mmole of 1-hydroxybenzotriazole:

Boc-Ser(Bzl), Boc-Met, Boc-Asp(chx), Boc-Gln, Boc-Leu, Boc-Lys(2-Cl-Z), Boc-Arg(Tos), Boc-Ala, Boc-Ser(Bzl), Boc-Leu, Boc-Gln, Boc-Ala, Boc-Leu, Boc-Val, Boc-Lys(2-Cl-Z), Boc-Arg(Tos), Boc-Tyr(2-Br-Z), Boc-Ser(Bzl), Boc-Ala, Boc-Thr(Bzl), Boc-Phe, Boc-Ile, Boc-Ala, Boc-Asp, Boc-Ala, Boc-Tyr(2-Br-Z). After removal of the last Boc group and washing and drying, the completed resin weighed 2.6 g.

6.1.4. $Ala^{8,15}$-GRF(1-29)NH_2

The resin described in Section 6.1.3. (2.6 g, 0.5 mmole) was subjected to cleavage with hydrogen fluoride and column purification as described in Section 6.1.2. Repeated lyophilization of the solution from water gives the desired product as a white, fluffy powder.

The product is found to be homogeneous by hplc and tlc. Amino acid analysis of an acid hydrolysate confirms the composition of the octapeptide.

In a similar manner, the following peptides were also made:

Aib^8, Ala^{15}-GRF(1-29)NH_2
$Ala^{8,15}$, Glu^{25}, $Leu^{25,26}$-GRF(1-29)NH_2
D-Ala^2, Leu^7, $Ala^{8,15}$, Glu^{25}, $Leu^{26,27}$-GRF(1-29)NH_2
D-Ala^{21}, $Ala^{8,15}$, Glu^{25}, $Leu^{26,27}$-GRF(1-29)NH_2
$Ala^{8,9,15}$, Glu^{25}, $Leu^{26,27}$-GRF(1-29)NH_2
D-Ala^2, $Ala^{8,15}$-GRF(1-29)NH_2
D-Ala^{21}, $Ala^{8,9,15}$-GRF(1-29)NH_2
$Ala^{7,8,9,15}$-GRF(1-29)NH_2
$Ala^{8,9,10,15}$-GRF(1-29)NH_2
D-Arg^2, $Ala^{8,1}$-GRF(1-29)NH_2

6.2. BIOLOGICAL ACTIVITY

A number of the peptides of the present invention were tested in an *in vitro* assay for their utility to stimulate relase of growth hormone from pituitary cells.

6.2.1. Pituitary Cell Dispersion

Anterior pituitaries from adult male rats weighing 200-250 g and housed under controlled conditions (lights on from 0500-1900 h), were dispersed using aseptic technique by a previously described trypsin/DNase method (Heiman *et al.*, *Endocrinology* 116:410-415, 1985) derived

from other methods (Ben-Jonathan, Meth. Enzymol. 103:249-257, 1983; Hoefer et al., Mol. Cell. Endocrinol. 35:229-235, 1984).

6.2.2. Cell Culture

The dispersed cells were diluted with sterile-filtered Dulbecco's modified Eagle medium (MEM) (Gibco Laboratories (GIBCO), Grand Island, NY), which was supplemented with 2.5% fetal calf serum (GIBCO), 3% horse serum (GIBCO), 10% fresh rat serum (stored on ice for no longer than 1 h) from the pituitary donors, 1% MEM nonessential amino acids (GIBCO), gentamycin (10 ng/ml; Sigma) and Nystatin (10,000 U/ml; GIBCO). The cells were counted with a hemacytometer (approximately 2,000,000 cells per pituitary) and randomly plated at a density of 200,000 cells per well (Costar cluster 24; Rochester Scientific, Rochester, NY). The plated cells were maintained in the above Dulbecco's medium in a humidified atmosphere of 95% air and 5% CO_2 at 37°C for 96 h.

6.2.3. In Vitro Incubation

In preparation for a hormone challenge, the cells were washed 3 X with medium 199 (GIBCO) to remove old medium and floating cells. Each dose of secretagogue (diluted in siliconized test tubes) was tested in quadruplicate wells in a total volume of 1 ml medium 199 containing 1% BSA (fraction V; Sigma Chemical, St. Louis, MO). Cells were pulsed in the presence of 0.1 nM somatostatin to maintain control levels within narrow limits and to increase the ratio of maximally stimulated levels to basal secretory levels without adding additional growth factors or glucocorticoids. After 3 h at 37°C in an air/carbon dioxide atmosphere (95/5%), the medium was removed and stored at -20°C until assayed for hormone content.

6.2.4. GH RIA

GH in plasma and media was measured by a standard double antibody RIA using components generously supplied by NIDDK and the National Hormone and Pituitary Program. University of Maryland School of Medicine.

6.2.5. Results

Potencies were calculated by 4-point assay according to the method described by Pugsley (Endocrinology 39:161-176, 1946). The results of these tests are presented in Table 1. As can be seen from these data, the combination of Ala at the 8 with Ala at the 15 position greatly increases the efficacy of the peptide relative to the analogue with Ala^{15} alone. Similarly, addition of an Ala^{9} and or $D\text{-}Ala^{2}$ also enhances the peptides GRF activity. On the other hand, substitution of Ala at other sites in the N-terminal region, i.e., at positions 7 and 10 have an essentially detrimental effect when combined with the $Ala^{8,9,15}$ combination.

TABLE 1

IN VITRO POTENCIES OF GRF (1-29)NH_2 ANALOGUES

Ala^{15}	5±1(4)
Ala^{8}	4±1(13)
$Ala^{8,15}$	15±2(15)
Aib^{8}, Ala^{15}	2±1(3)
$Ala^{8,15}$, Glu^{25}, $Leu^{26,27}$	7±2(5)
$D\text{-}Ala^{2}$, Leu^{7}, $Ala^{8,15}$, Glu^{25}, $Leu^{26,27}$	0.3±0.2(5)
$D\text{-}Ala^{2}$, $Ala^{8,15}$, Glu^{25}, $Leu^{26,27}$	7±1(4)
$Ala^{8,9,15}$, Glu^{25}, $Leu^{26,27}$	33±9(6)
$D\text{-}Ala^{2}$, $Ala^{8,15}$	27±10(8)
$D\text{-}Ala^{2}$, $Ala^{8,9,15}$	49±14(8)
$Ala^{7,8,9,15}$	2±1(2)
$Ala^{8,9,10,15}$	1±1(2)

Potencies were calculated by 4-point assay. Values are the means ± sem. The value in parentheses is the number of independent experiments using multiple doses of analogue used in calculating the mean potency. A GRF(1-29)NH_2 standard (potency=1) was contained in each assay. For further details, see Murphy and Coy, Peptide Research 1(1):36-41(1988).

WHAT WE CLAIM IS:

1. Peptides comprising the sequence

$R_1 - R_2 - R_3 - Ala - Ile - Phe - R_7 - R_8 - R_9 - R_{10} - Arg -$
$R_{12} - R_{13} - R_{14} - R_{15} - Gln - R_{17} - R_{18} - Ala - Arg - R_{21} -$
$Leu - R_{23} - R_{24} - R_{25} - R_{26} - R_{27} - R_{28} - R_{29}$

wherein R_1 is des-amino-Tyr, or A-R_1, in which A is lower alkyl, lower cycloalkyl, benzyl or lower acyl and R_1 is Tyr, D-Tyr, Met, Phe, D-Phe, pCl-Phe, Leu, His, or D-His with or without a $C^{\alpha}Me$ or $N^{\alpha}Me$ substituent

R_2 is Ala, D-Ala, D-NMA, or D-Arg;
R_3 is Asp or D-Asp;
R_7 is Thr, Aib, Leu, Trp, β-Nal, or p-X-Phe, in which X = H, F, Cl, Br, NO_2 or Me;
R_8 is Ala, Aib, Leu, Trp, β-Nal, or p-X-Phe, in which X is H, F, Cl, Br, NO_2 or Me;
R_9 is Ser, Ala, Aib, Leu, Trp, β-Nal or p-X-Phe, in which X is H, F, Cl, Br, NO_2 or Me
R_{10} is Tyr or D-Tyr
R_{12} and R_{21} are Lys, Arg, or N^{ξ}-B-Lys, in which B is lower alkyl or cycloalkyl
R_{13} is Ile or Val
R_{14} is is Leu or D-Leu
R_{15} Gly, Ala, Leu, Asn, Gln or Aib
R_{17} is Leu or D-Leu
R_{18} is Tyr or Ser
R_{23} is Leu or d-Leu
R_{24} His or Gln
R_{25} is Glu, Asp, D-Glu or D-Asp
R_{26} is Ile or Leu
R_{27} is Met, D-Met, Ala, Nle, Ile, Leu, Nva, or Val
R_{28} is Asn, Ser or des R_{28}
R_{29} is Arg, D-Arg or des R_{29};

and pharmaceutically acceptable salts thereof.

2. The peptides of Claim 1 in which R_8 is Ala.

3. The peptide of Claim 2 in which R_{15} is Ala.

4. The peptide of Claim 3 in which R_9 is Ala.

5. The peptide of any one of Claims 1-4 in which R_2 is D-Ala.

6. The peptide of Claim 4 in which R_{25} is Glu.

7. The peptide of Claim 6 in which each of R_{26} and R_{27} is Leu.

8. The peptide of Claim 5 in which R_{25} is Glu.

9. The peptide of Claim 5 in which each of R_{26} and R_{27} is Leu.

10. The peptide of Claim 1 which comprises Ala^8-GRF (1-29).

11. The peptide of Claim 1 which comprises $Ala^{8,15}$-GRF (1-29).

12. The peptide of Claim 1 which comprises $Ala^{8,9,15}$-GRF (1-29).

13. The peptide of Claim 1 which comprises D-Ala^2, $Ala^{8,15}$-GRF (1-29).

14. The peptide of Claim 1 which comprises D-Ala^2, $Ala^{8,9,15}$-GRF (1-29).

15. The peptide of Claim 1 which comprises D-Ala^2, Ala^{8,15}, Glu^{25}, Leu^{26,27}-GRF (1-29).

16. The peptide of Claim 1 in which R_2 is D-Arg.

17. The peptide of Claim 16 in which R_2 is Ala.

18. The peptide of Claim 16 in which R_{15} is Ala.

19. The peptide of Claim 17 in which R_{15} is Ala.

20. The peptide of Claim 19 in which R_9 is Ala.

21. The peptide of Claim 20 in which R_{25} is Glu.

22. The peptide of Claim 21 in which each of R_{26} and R_{27} is Leu.

23. The peptide of Claim 16 which comprises D-Arg^2, Ala^8-GRF (1-29).

24. The peptide of Claim 16 which comprises D-Arg^2, Ala^{8,15}-GRF (1-29).

25. The peptide of Claim 16 which comprises D-Arg^2, Ala^{8,9,15}-GRF (1-29).

26. A pharmaceutical composition comprising an effective amount of a peptide comprising the sequence $R_1 - R_2 - R_3 - Ala - Ile - Phe - R_7 - R_8 - R_9 - R_{10} - Arg - R_{12} - R_{13} - R_{14} - R_{15} - Gln - R_{17} - R_{18} - Ala - Arg - R_{21} - Leu - R_{23} - R_{24} - R_{25} - R_{26} - R_{27} - R_{28} - R_{29}$

wherein R_1 is des-amino-Tyr, or A-R_1, in which A is lower alkyl, lower cycloalkyl, benzyl or lower acyl and R_1 is Tyr, D-Tyr, Met, Phe, D-Phe, pCl-Phe, Leu, His, or D-His with or without a $C^{\alpha}Me$ or $N^{\alpha}Me$ substituent

R_2 is Ala, D-Ala, D-NMA, or D-Arg;
R_3 is Asp or D-Asp;
R_7 is Thr, Ala, Aib, Leu, Trp, β-Nal, or p-X-Phe, in which X = H, F, Cl, Br, NO_2, or Me;
R_8 is Ala, Aib, Leu, Trp, β-Nal, or p-X-Phe, in which X is H, F, Cl, Br, NO_2, or Me;
R_9 is Ser, Ala, Aib, Leu, Trp, β-Nal or p-X-Phe, in which X is H, F, Cl, Br, NO_2, or Me
R_{10} is Tyr or D-Tyr
R_{12} and R_{21} are Lys, Arg, or N^{ϵ}-B-Lys, in which B is lower alkyl or cycloalkyl
R_{13} is Ile or Val
R_{14} is is Leu or D-Leu
R_{15} Gly, Ala, Leu, Asn, Gln or Aib
R_{17} is Leu or D-Leu
R_{18} is Tyr or Ser
R_{23} is Leu or d-Leu
R_{24} His or Gln
R_{25} is Glu, Asp, D-Glu or D-Asp
R_{26} is Ile or Leu
R_{27} is Met, D-Met, Ala, Nle, Ile, Leu, Nva, or Val
R_{28} is Asn, Ser or des R_{28}
R_{29} is Arg, D-Arg or des R_{29};

and pharmaceutically acceptable salts thereof, in combination with a pharmaceutically acceptable carrier.

27. The composition of Claim 26 in which R_8 is Ala.

28. The composition of Claim 27 in which R_{15} is Ala.

29. The composition of Claim 29 in which R_9 is Ala.

30. The composition of any one of Claims 26-29 in which R_2 is D-Ala.

31. The composition of Claim 29 in which R_{25} is Glu.

32. The composition of Claim 29 in which each of R_{26} and R_{27} is Leu.

33. The composition of Claim 30 in which R_{25} is Glu.

34. The composition of Claim 30 in which each of R_{26} and R_{27} is Leu.

35. The composition of Claim 26 in which the peptide comprises Ala^{8}-GRF(1-29).

36. The composition of Claim 26 in which the peptide comprises $Ala^{8,15}$-GRF(1-29).

37. The composition of Claim 26 in which the peptide comprises $Ala^{8,9,15}$ GRF(1-29).

38. The composition of Claim 26 in which the peptide comprises D-Ala^{2}, Ala^{8}-GRF(1-29).

39. The composition of Claim 26 in which the peptide comprises D-Ala^{2}, $Ala^{8,5}$-GRF(1-29).

40. The composition of Claim 26 in which the peptide comprises D-Ala^2, D-Ala^2 Ala^{8,9,15} GRF(1-29).

41. The composition of Claim 26 in which the peptide comprises D-Ala^2, Ala^{8,15}, Glu^{25}, Leu^{26,27} GRF(1-29).

42. The composition of Claim 26 in which R_2 is D-Arg.

43. The composition of Claim 42 in which R_8 is Ala.

44. The composition of Claim 26 in which R_{15} is Ala.

45. The composition of Claim 42 in which R_{15} is Ala.

46. The composition of Claim 45 in which the R_9 is Ala.

47. The composition of Claim 46 in which the R_{25} is Glu.

48. The composition of Claim 47 in which each of R_{26} and R_{27} is Leu.

49. The composition of Claim 42 in which the peptide comprises D-Arg^2, Ala^8 GRF(1-29).

50. The composition of Claim 42 in which the peptide comprises D-Arg^2, Ala^{8,15} GRF(1-29).

51. The composition of Claim 42 in which the peptide comprises D-Arg^{2}, Ala^{8,9,15} GRF(1-29).

52. A method of increasing production of growth hormone in an individual in need of such treatment which comprises administering to the individual an effective amount of the peptide of Claim 1.

53. The method of Claim 52 in which the individual is a mammal.

54. The method of Claim 53 in which the individual is human.

55. The method of any one of Claims 52-54 in which the dosage is from about 5 μg - 5 μg/kg of body weight.

56. The method of Claim 52 in which the peptide is used to treat pituitary dwarfism.

57. The method of Claim 52 in which the peptide is used to enhance milk production in livestock.

58. The method of Claim 52 in which the peptide is used to enhance growth of livestock.

59. The method of Claim 52 in which the peptide is used to enhance the ratio of protein to fat in an animal.

60. The method of any one of Claims 58-60 in which the livestock is cattle or pigs.

61. A method for decreasing the production of growth hormone in an individual in need of such treatment which comprises administering to the individual an effective amount of the peptide of Claim 16.

62. The method of Claim 61 in which the individual is human.

63. The method of Claim 61 or 62 in which the dosage is from about 5 μg - 5 μg/kg of body weight.

64. The method of anyone of Claims 62-64 in which the peptide is used to treat acromegaly.

INTERNATIONAL SEARCH REPORT

International Application N PCT/US91/03053

I. CLASSIFICATION OF SUBJECT MATTER (if several classification symbols apply, indicate all) [6]

According to International Patent Classification (IPC) or to both National Classification and IPC

IPC(5): A61K 37/43; C07K 7/10
U.S.A.: 514/2; 530/324

II. FIELDS SEARCHED

Minimum Documentation Searched [7]

Classification System	Classification Symbols
U.S.	514/12; 530/324

Documentation Searched other than Minimum Documentation to the Extent that such Documents are Included in the Fields Searched [8]

III. DOCUMENTS CONSIDERED TO BE RELEVANT [9]

Category *	Citation of Document, [11] with indication, where appropriate, of the relevant passages [12]	Relevant to Claim No. [13]
Y	US, A, 4,649,131, (Felix et al.) 10 March 1987, see col. 1, lines 53-64, col. 2, lines 1-29.	1-64
Y	US, A, 4,689,318 (Kaiser et al.) 25 August 1987, see abstract.	1-64
Y	Biochemical and Biophysical Research Communications, vol. 49, no. 2, issued 16 December 1987, Sato et al., "Synthesis and in vitro bioactivity of human growth hormone-releasing factor analogs substituted with a single D-amino acid", pp. 531-537 see page 535 table 3.	1-25
Y	Peptide: Chem. Biol., Proceedings American Peptide Symposium, 10th Meeting, issued 1987, Felix et al., "Synthesis and biological activity of novel linear and cyclic GRF analogs", pp 465-467 see page 466, table 1.	1-25

* Special categories of cited documents: [10]

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

IV. CERTIFICATION

Date of the Actual Completion of the International Search

05 August 1991

Date of Mailing of this International Search Report

20 AUG 1991

International Searching Authority

ISA/US

Signature of Authorized Officer

T. D. Wessendorf

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